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TECHNICAL MANUSCRIPT 34

RIFT VALLEY FEVER VIRUS HEPATITIS

LIGHT AND ELECTRON MICROSCOPIC STUDIES
IN THE MOUSE

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ABSTRACT

Rift Valley fever virus produces a necrotizing hepatitis in mice that is similar to certain other virus hepatitides. The virus infects the hepatic parenchymal cells and new virus is formed in the cytoplasm within a membrane-limited system resembling the Golgi apparatus. Unique structural alterations of the ergastoplasm are associated with this process and may be manifestations of metabolic dysfunction. The acidophilic nuclear inclusion is not formed of virus matrix or virus particles and may represent a degenerative phenomenon. Changes in the Kupffer cells follow infection of the parenchymal cells.

I. INTRODUCTION

Rift Valley fever virus (RVFV) infects a wide range of species, including man.¹ Total hepatic necrosis is seen in the highly susceptible species, e.g., the lamb, mouse, and hamster.² Under natural conditions or when these animals are injected with minimal doses, the lesions are initially focal, gradually enlarge and increase in number, and finally, between 60 and 90 hours, all of the hepatic parenchymal cells become necrotic in a precipitous manner. Mims showed that in mice inoculated intravenously with very large doses of RVFV, a synchronous cycle of infection and necrosis occurred within 10 hours.³ We chose to study RVFV in a similar system in order to minimize sampling errors and to determine whether the cells were actually infected, where the virus was produced, and the significance of the nuclear inclusion.

II. MATERIALS AND METHODS

The source, production, and assay of RVFV were as previously described.⁴ Thirty-six 10- to 12-gram Swiss-Webster mice were inoculated intravenously with 0.5 milliliter of lamb serum containing $1 \times 10^{9.7}$ mouse intraperitoneal median lethal doses (MIPLD₅₀) of RVFV. Fourteen mice were inoculated with 0.5 milliliter of normal lamb serum. Two infected and one control mouse were sacrificed at time zero and at hourly intervals for 12 hours. The remaining infected mice were observed until they died.* Samples of blood, liver, and lung from each mouse were assayed for virus. Portions of the liver were frozen and the remainder was fixed in cold buffered formalin, Carnoy's fixative (6:3:1), absolute methanol, acetone, or one per cent osmium tetroxide in White's saline at pH 7.2. The frozen and acetone-fixed tissues were used in fluorescent antibody studies. The osmium-fixed tissues were dehydrated in ethanol and propylene oxide, and embedded in Epon 812. They were then cut with glass knives on a Porter-Blum microtome, stained with uranyl acetate, and examined with an RCA EMU 3F microscope at 50 kilovolts. The remaining tissues were processed through paraffin, cut at four microns, and stained with hematoxylin and eosin, methyl green pyronin, and with periodic acid-Schiff method with and without prior diastase digestion, the Feulgen method, and the method for demonstrating the Millon reaction. Frozen sections were stained with 0.1 per cent acridine orange at pH 3.6 for 10 minutes and with a globulin fraction, derived from the serum of sheep that had survived RVFV infection, conjugated with fluorescein isothiocyanate.

* In conducting the research reported herein, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

III. RESULTS

The injected mice died between 14 and 18 hours after inoculation and manifested total hepatic necrosis associated with mild hemorrhagic manifestations. No morphological evidence of infection or necrosis was found in other organs. The control mice did not die and showed no changes at necropsy.

A. VIRUS ASSAY

The mean values of the virus titers in the blood, liver, and lung from the two mice sacrificed at each point are shown in Figure 1.* The concentration of virus in the blood exceeded that in the liver from the second through the fourth hour and was essentially the same thereafter.

B. LIGHT MICROSCOPY

The hepatic parenchymal cells underwent a synchronous sequence of alterations. Glycogen depletion was rapid and complete by the sixth hour, even though the mice had not been fasted and were left on food and water. As the glycogen content decreased, the clumping of the ergastoplasm diminished, as indicated by basophilia, pyroninophilia, or of red fluorescence in acridine-orange-stained sections. From the seventh to the tenth hour the ribonucleoprotein content of the cytoplasm decreased, and by the twelfth hour only faint staining was seen in a few cells. No increase in lipid was found. Between the eighth and twelfth hours, the cells separated from each other and erythrocytes became enmeshed, first singly, then in groups of two or more, and then in clusters in the interstices. Some cells became hyalinized but the majority disintegrated, leaving a mixture of necrotic but recognizable cells, cellular debris, and erythrocytes. There was significant absence of inflammatory cells (Figure 2). Microthrombi containing cellular debris, presumably hepatic in origin, were found in the small pulmonary arteries.

The nuclear chromatin was margined distinctly by the eighth hour and, thereafter, a pale eosinophilic inclusion appeared in the nuclear sap. The inclusions failed to stain for deoxyribonucleic acid (DNA), ribonucleic acid (RNA), virus antigen, basic protein, mucopolysaccharide, or glycogen (Figures 3 and 4). We did not find any change in nucleolar staining at any time.

* Original photomicrographs are filed in Pathology Division Office.

LEVELS OF RIFT VALLEY FEVER VIRUS
IN MICE FOLLOWING IV INOCULATION OF
 1×10^{10} MID₅₀ OF RVFV

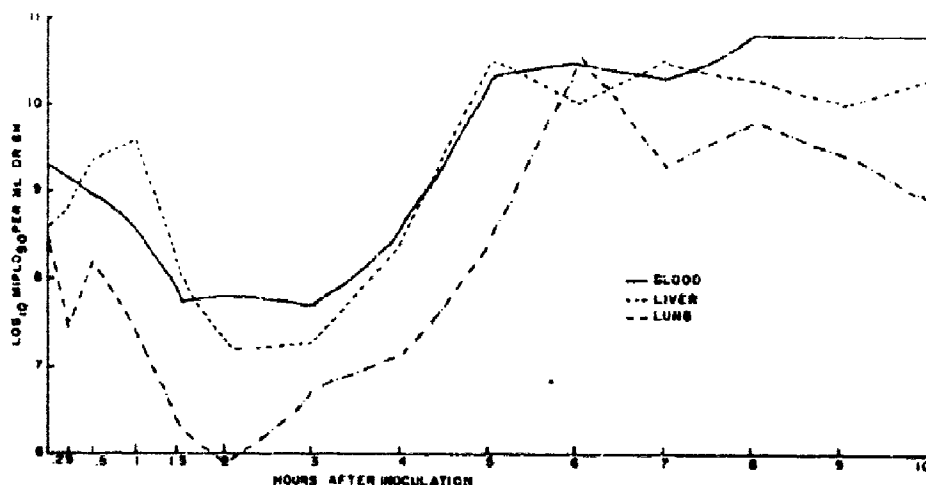


Figure 1. Mean Values of Virus Titers in Sacrificed Mice.

Excessive nonspecific staining by the fluorescein-conjugated globulin was encountered, in spite of repeated extractions and changes in procedure. On the few occasions when the controls indicated satisfactory specificity, distinct perinuclear cytoplasmic staining was found. At no time was staining in the nuclei observed, nor in the Kupffer cells. In experiments reported elsewhere,⁵ specific immunofluorescence was not found in the nuclei of lamb liver cells nor in tissue cultures of hamster kidney and Chang human liver cells infected with RVFV.

C. ELECTRON MICROSCOPY

The mode of attachment or ingress of the virus was not observed. After the first hour, small aggregates of oval and round particles appeared in dilated endoplasmic sacs and lacunae (Figures 5 through 9).

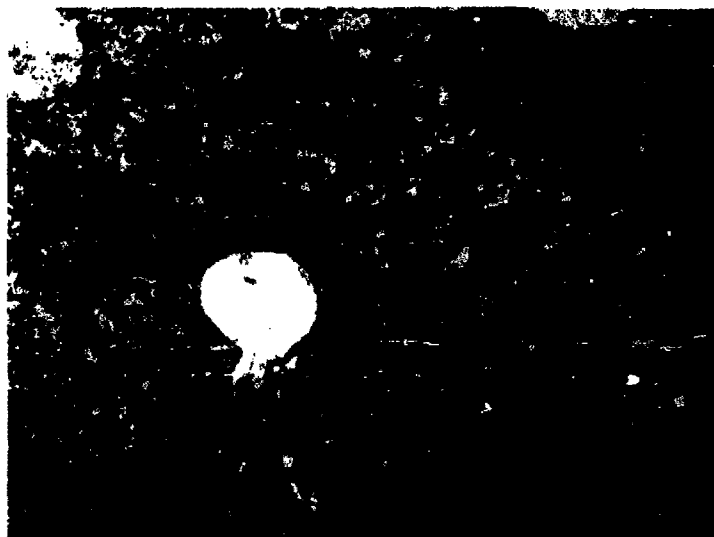


Figure 2. Necrotic Mouse Liver 12 Hours after Infection with a Massive Dose of RVFV. Hematoxylin and eosin, 150X.

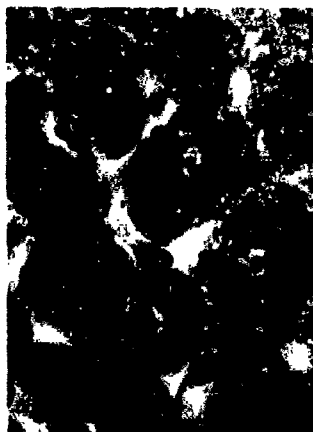


Figure 3. Nuclear Alteration 8 to 10 Hours after Infection. The chromatin is distinctly margined, leaving a pale central sphere in which an irregularly shaped, faintly eosinophilic inclusion is found. Hematoxylin and eosin, 1000X.



Figure 4. Section Adjacent to that Shown in Figure 2, Stained by the Feulgen Reaction. The nuclear inclusion does not react for DNA. It is stained by the light green. Feulgen, 1250X.



Figure 5. Many Incompletely Formed Viruses (arrows) are Present Within a Membrane-Limited Complex Two Hours after infection. The pale areas (gl) toward the left and bottom are deposits of glycogen that have been leached during fixation. A portion of the liver cell nucleus (n) is seen at the upper left, and to the right is a mitochondrion (m). Approximately 31,000X.

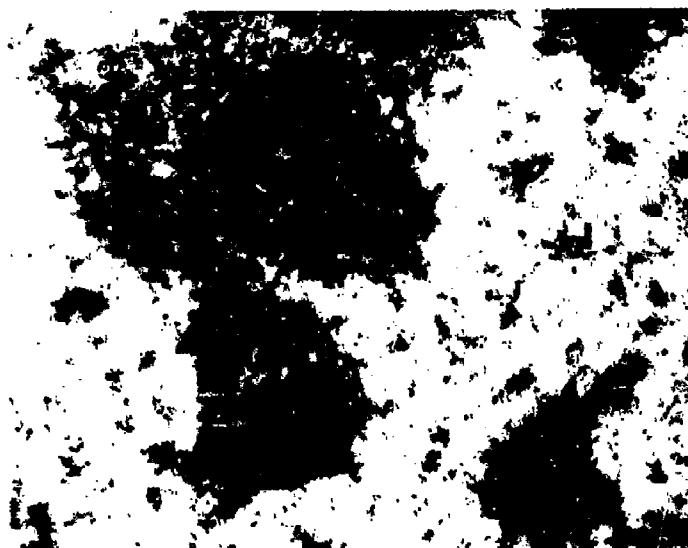


Figure 6. The Oyal and Round Shapes of the Forming Viruses are Seen in the Vesicles (arrow) beneath the Mitochondrion (m). Approximately 31,000X.



Figure 7. The Varying Sizes of the Viruses and the Agranularity of the Limiting Membrane (arrow) are Shown. Approximately 35,000X.

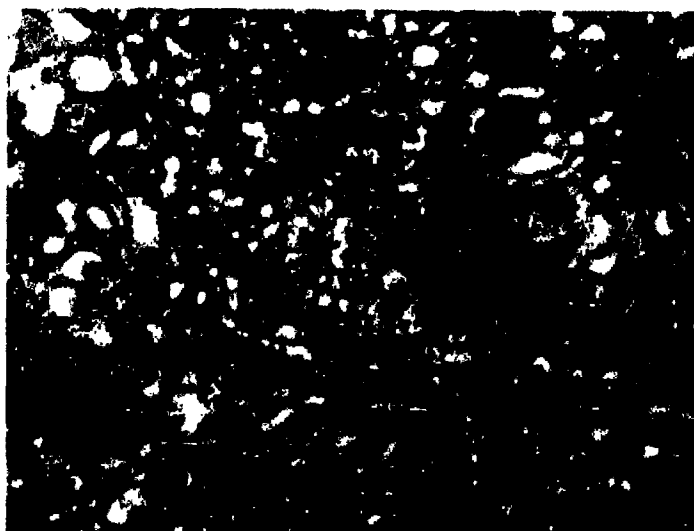


Figure 8. Four Hours after Infection some Viruses have a Distinct Membrane and Nucleoid (v), others Remain Amorphous (vl). Approximately 30,000X.

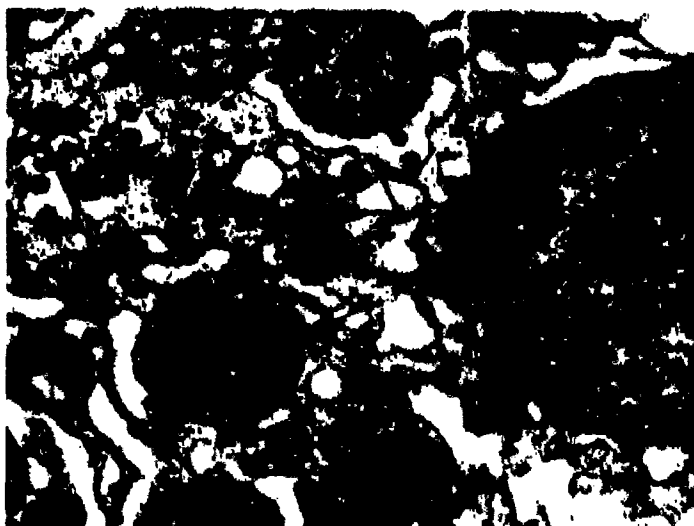


Figure 9. Six Hours after Infection some Viruses are still Present. The ribosomes are fewer, the mitochondrial matrix (m) flocculated and fine lamellar alteration of the endoplasmic reticulum is noted near the nucleus (arrows). Approximately 30,000X.

This was not preceded by the condensation of precursor particles on the outer surface of the membranes lining these spaces, nor were particles seen crossing these membranes. Initially the bodies were relatively electron-clear, but later appeared to condense and develop a distinct limiting membrane and dense nucleoid. The initial amorphous particles varied from 70 to 120 millimicrons in diameter. When fully formed, the virus measured approximately 90 millimicrons in diameter. A thin section of RVFV, centrifuged from lamb serum at 100,000g for one hour, is shown in Figure 13. It has the same morphology and size. Vesicles containing virus appeared to migrate toward the plasma membrane. The walls of these vesicles then fused with the plasma membrane and the mature virus was released into the space of Disse. The virus did not acquire another membrane, nor did it lose one during the process of release.

This cycle occurred rapidly, so that after six to eight hours it was difficult to find virus within the cells (Figures 10 to 12). Neither virus nor aggregates of amorphous matrix were found within the nucleus, nor did the nucleoli enlarge.

Depletion of glycogen and ribosomes was recognizable by three hours and continued. Aggregates of smooth-surfaced endoplasmic reticulum appeared, as well as an unusual form of the ergastoplasm. Normally the ergastoplasmic membranes are studded with ribosomes on their outer surface. The distance between adjacent membranes is variable, and within the space unattached ribosomes are seen. The alteration found in the RVFV-infected liver cells is illustrated in Figures 14 through 17. Adjacent ergastoplasmic membranes were uniformly approximated, the distance between them averaging 100 Å. In this space a relatively regular banding appeared. These cross-bars were spaced at intervals of 100 to 200 Å. In some areas, the banded or ladder-like pattern merged into a fine laminae fraying or stacking of less well-defined membranes. These variations are probably the result of differing planes of section. It was concluded that the composite structure that will account for them is composed of shelf-like cross-bands, rather than isolated granules or particles, at right angles to the approximated ergastoplasmic membranes. The ergastoplasmic sacs were dilated in these areas and electron-clear. Although the density of their matrix increased progressively, the mitochondria remained intact until the cells disintegrated. As the cells shrank they separated from each other, except at the parabiliary desmosomes (Figure 16). Finally, distinct myelin figures appeared and the plasma membranes ruptured. No increase in dense bodies preceding the dissolution of these cells was observed.

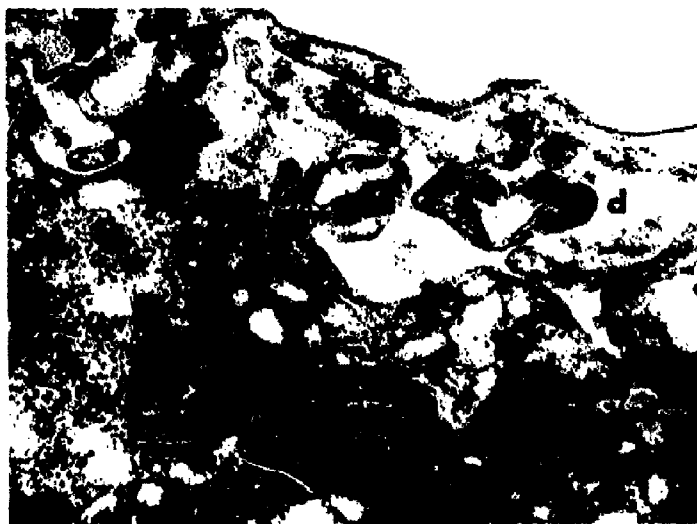


Figure 10. Four Viruses (arrows) are Present in the Space of Disse (d) Between the Liver Cell Microvilli and Intact Kupfer cell Cytoplasm (K). Approximately 57,000X.

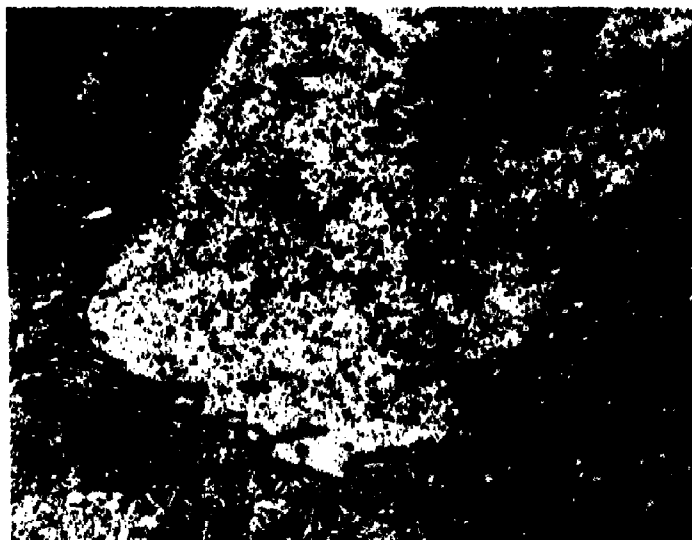


Figure 11. Collection of Virus (v) in the Extracellular Space Seven Hours after Infection. Approximately 14,000X.



Figure 12. Three Viruses in the Space of Disac. The one on the left is superimposed on the tip of a microvillus and has a distinct hexagonal nucleoid. The plasma membrane is indistinct beneath the middle one, and the one on the right overlies the base of a microvillus (see text). Approximately 80,000X.

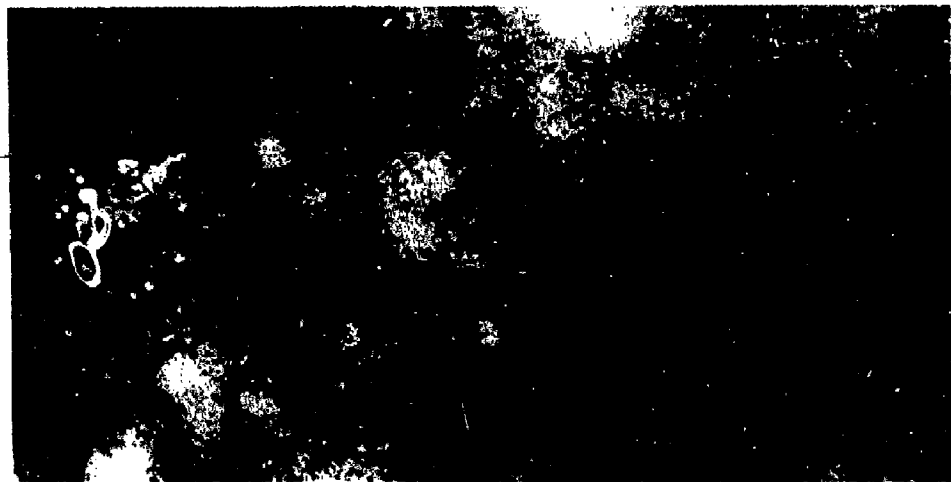


Figure 13. A Thin Section of a RVFV Centrifuged from Lamb Serum. It has the same characteristics as those shown in Figure 10. Approximately 88,000X.



Figure 14. The Aberration of the Endoplasmic Reticulum Found Six to Nine Hours after Infection. In some places it is finely laminar, (l), in others it has a regular cross-banding (b) between adjacent membranes. Approximately 44,000X.



Figure 15. An Example of the Concentric Form of the Ergastoplasmic Change that encircles a Mitochondrion (m). At the tip of the arrow (b) eight pairs of banded membranes are seen. Approximately 60,000X.

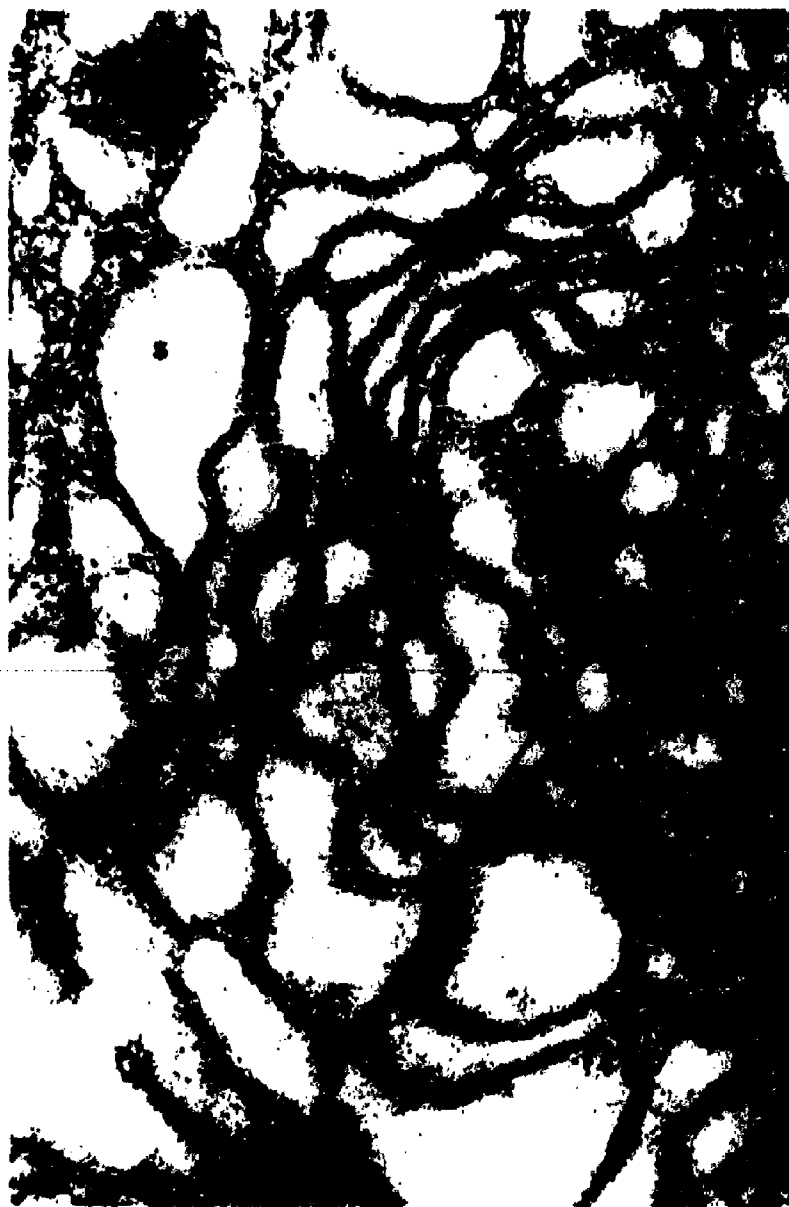


Figure 16. A Detailed View of the Alteration in the Ergastoplasm in which, at the Tip of the Arrow Marked (h), Three Pairs of Cross-banded Membranes are seen. Followed toward the bottom of the plate, they merge into the laminar pattern, arrow (l). The cross-bars are smaller than the ribosomes (r). The right wall of the ergastoplasmic sac (s) is formed of banded membranes; the left is unaltered. Approximately 80,000X.

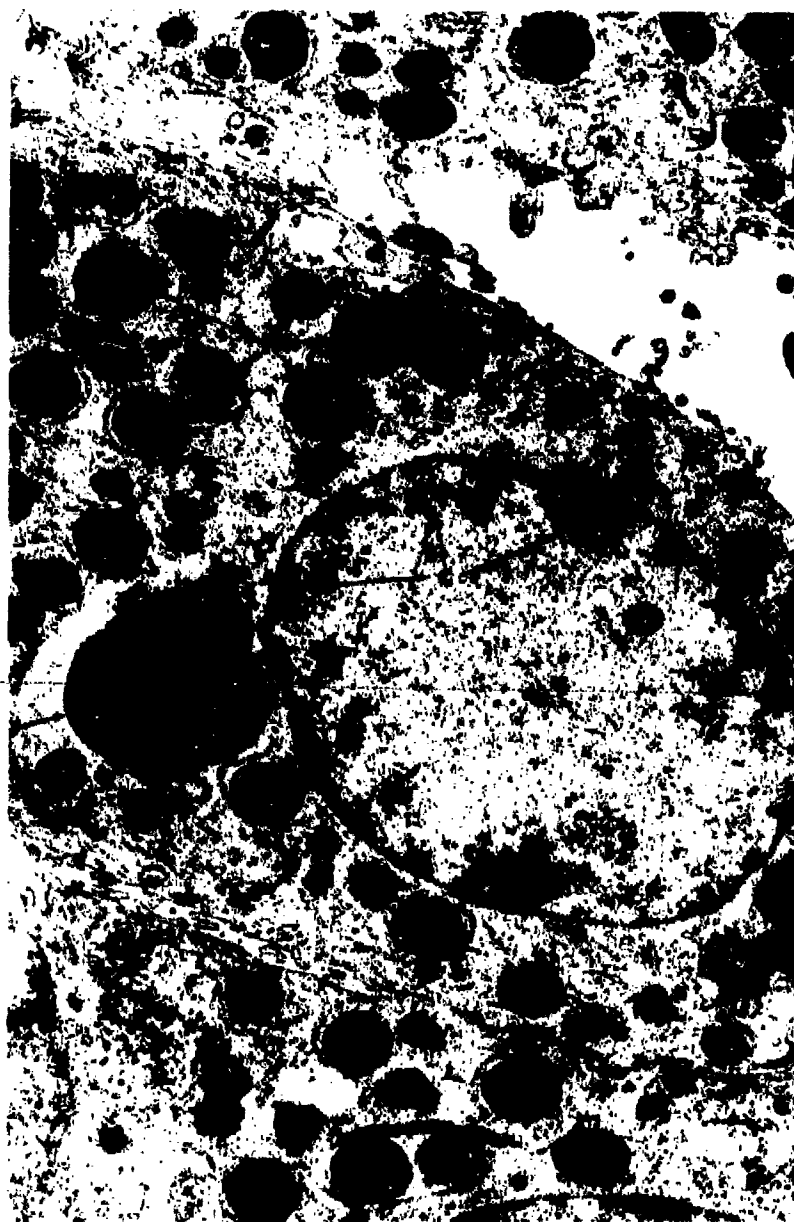


Figure 17. The Dying Liver Cell, Eight Hours after Infection, is devoid of Virus and Depleted of Glycogen and Ergastoplasm. A few ribosomes are scattered between the remaining membranous components. The mitochondria (m) are intact. Their matrix is exceptionally dense. The chromatin (c) is margined and the nuclear sap faintly granular. A portion of an erythrocyte (e) is present to the left of the nucleus. Approximately 11,000X.

IV. DISCUSSION

The mechanisms, whereby viruses kill cells are poorly understood. In some instances, particularly when large doses are used, toxic destruction without apparent infection or viral multiplication occurs. The results of the assay (Figure 1) and the electron microscopic findings of multiplication indicate that the hepatic necrosis produced by RVFV is not merely a toxic phenomenon. Another hypothesis suggests that the virus-infected cell is forced to transfer its anabolic functions into virus production so completely that it becomes "exhausted" and dies. The condition in RVFV hepatitis might fit this latter hypothesis, although the amount of virus estimated as synthesized by the liver is hardly sufficient to exhaust these active cells. It is more probable that this type of virus infection irrevocably alters vital enzymatic systems, as indicated by Ginsberg⁶ and Jones and Cohen.⁷ The aberration of the ergastoplasm described may be a structural manifestation of some of the metabolic changes associated with virus production or its aftermath.

Rift Valley fever virus is formed in a manner that differs from that described for certain other animal viruses.^{8,9} It forms within a membrane-lined system that is generally agranular and thus resembles the Golgi apparatus. We may assume that the virus nucleic acid, as well as the coat proteins and lipids, is formed in the ergastoplasm and transported to the Golgi apparatus wherein the virus is assembled. Thereafter, the egress of the virus is similar to that of secretory products of exocrine cells. It does not bud from the cell surface as do the virus of influenza and many of the tumor viruses.

An increasing number of reports on experimental virus hepatitis are available. These include studies on yellow fever,¹⁰⁻¹² ectromelia (mouse pox),¹³ and a series of papers on the mouse hepatitis viruses (MHV).^{7,14,15} The pitfalls of sampling, particularly in asynchronous systems, and the difficulties of differentiating changes that are antecedent and causally related to virus multiplication from those that are subsequent and probably degenerative are well illustrated. Even within an apparently synchronous system, such as used in this study, the limitations of sampling and, perhaps, the rapidity of multiplication prevented the finding of virus in each cell. It may only be surmised that all the cells were infected by noting the comparable secondary changes.

Tigertt *et al.*,¹⁰ Rubner and Miyai,¹⁴ and Jones and Cohen⁷ have noted changes in the Kupffer cells that precede the appearance of histological damage in the liver cells. They have suggested that Kupffer cell damage may be the primary event and that hepato-cellular necrosis is secondary. Similar changes in the Kupffer cells were found at two to three hours after infection in this study. However, this did not precede the appearance of virus infection of the parenchymal cells as seen by electron microscopy. Infected Kupffer cells were not found. Thus, it is difficult to accept the hypothesis that hepatic necrosis is secondary to primary injury to the Kupffer cells.

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